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I further certify that the above application is now proceeding in the name of HOWARD FLOREY INSTITUTE OF EXPERIMENTAL PHYSIOLOGY AND MEDICINE pursuant to the provisions of Section 113 of the Patents Act 1990.

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DAVID DANIEL CLARKE
ASSISTANT DIRECTOR PATENT SERVICES

AUSTRALIA

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PROVISIONAL SPECIFICATION

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Applicant(s): ~~UNIVERSITY OF MELBOURNE~~
Howard Florey Institute of Experimental Physiology and Medicine

Invention Title: NEUROACTIVE PEPTIDE

The invention is described in the following statement:

NEUROACTIVE PEPTIDE

This invention relates to neuroactive peptides, and in particular to peptides which have the ability to act as analogues of angiotensin IV. The peptides of the invention bind with high affinity and specificity to a variety of sites in the central nervous system, and are useful as modulators of motor and cognitive function, and of neuronal development.

Background of the Invention

The renin-angiotensin system has diverse roles in the regulation of body fluid and electrolyte balance and blood pressure control. These actions are exerted in a variety of target organs, including the cardiovascular system, adrenal glands, kidney and central and peripheral nervous systems, by both the circulating hormone and hormone locally produced in tissues. Most of these actions are exerted by the octapeptide, angiotensin II, although the C-terminal heptapeptide angiotensin III has some activity. The hexapeptide

NH2-Val Tyr Ile His Pro Phe-COOH,

corresponding to the 3-8 fragment of angiotensin II (*ie*. amino acids 3-8), is also called angiotensin IV (Ang IV), and has until recently been believed to be an inactive degradation product devoid of biological activity.

However, Harding and co-workers have confirmed an earlier report (Braszko *et al*, 1988) that Ang IV has central nervous system activity, and can modify learning and behaviour (Wright *et al*, 1995). In addition, Ang IV has vasoactive effects, and can dilate cerebral arteries (Haberl *et al*, 1991) and increase renal blood flow (Swanson *et al*, 1992). This, coupled with the discovery of highly

specific, high affinity sites for Ang IV binding in bovine adrenal and other tissues, has reawakened interest in the hexapeptide, and the subject has been comprehensively reviewed (Wright et al 1995).

5   Ang IV has been associated with the central nervous system effects of increasing stereotypy behaviour (Braszko et al, 1988) and facilitating memory retrieval in passive avoidance studies (Braszko et al, 1988; Wright et al, 1995). Ang IV also dilates cerebral arterioles (Haberl

10 et al, 1991), and increases renal blood flow (Swanson et al, 1992).

Receptor autoradiographic studies have revealed a widely abundant but selective and characteristic distribution of binding sites for $[^{125}I]$Ang IV in the

15 guinea pig, sheep and monkey central nervous systems, in regions associated with cholinergic neurons and in somatic motor and sensory associated areas (Miller-Wing et al, 1993; Moeller et al, 1995). In addition, Ang IV binding sites are abundant in supraspinal components of the

20 autonomic nervous system, and in the spinal cord are found in sympathetic preganglionic neurons, in the dorsal root ganglia, and in Lamina II of the dorsal horn, and in the motor neurons of the ventral horn (Moeller et al, 1995).

The distribution of the Ang IV binding site

25 differs from the localization of the Ang II AT_1 or AT_2 receptors. In addition, the pharmacology of each receptor is distinct in that the Ang IV site exhibits a low to very low affinity for $[Sar^1Ile^8]$Ang II, the non-subtype selective Ang II antagonist, and losartan (du Pont-Merck)

30 and PD 123319 (Parke-Davis), the specific AT_1 and AT_2 receptor antagonists respectively (Miller-Wing et al, 1993; Swanson et al, 1992; Hanesworth et al, 1993). Conversely,

Ang II receptors show a low affinity for the Ang IV binding site (Bennett and Snyder, 1976).

The wide distribution of the Ang IV binding site in motor, sensory and cholinergic regions suggests important roles for this peptide in the central nervous system. However, a physiological action of the peptide in neurons has yet to be clearly defined.

Numerous neurotransmitters and neuropeptides have been associated with the regulation of neuronal development. Acetylcholine inhibits neurite outgrowth from embryonic chicken ciliary ganglion cells and sympathetic neurons (Pugh and Berg, 1994; Small et al, 1995), and rat hippocampal neurons (Muttson, 1988). Conversely, vasoactive intestinal peptide stimulates superior cervical ganglion branching (Pincus et al, 1990) and somatostatin increases neuronal sprouting from Helisoma buccal ganglion neurons (Bulloch, 1987).

We have now surprisingly found that the peptide LVV-haemorphin 7, derived from β-globin, acts as an agonist of AngIV, and is the endogenous ligand for AngIV receptors in the brain. We have characterised its pharmacological activity. This enables us to design novel agonists and antagonists of AngIV action.

Summary of the Invention

In one aspect, the invention provides a neuroactive peptide having at least one of the biological activities of angiotensin IV as herein defined, comprising the amino acid sequence:

Leu-Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe,

or a biologically-active analogue or fragment of said peptide.

It will be clearly understood that the sequence of the invention may be modified by conservative amino acid substitutions, insertions, deletions or extensions, provided that the biological activity is retained. Such variants may, for example, include sequences comprising D-amino acids, non-naturally occurring amino acids, and/or amino acid analogues.

In an alternative aspect, the invention provides a non-peptide analogue of the peptide of the first aspect of the invention. This non-peptide analogue is to be understood to encompass modifications or substitutions of the peptide structure which are designed to improve the bioavailability, metabolic stability, half-life in the body, or to modify the biological activity, of the compound of the invention. Such non-peptide analogues are known in the art, for example compounds in which the peptide backbone is replaced by a non-peptide chain, and are often referred to as peptidomimetic compounds. Alternatively, in one or more of the peptide linkages the order of the nitrogen and carbon atoms can be reversed to form a pseudo peptide bond. One or more of the amino acid side-chains may be replaced by an analogous structure of greater stability. Many other such variations will occur to the person skilled in the art. The only requirement is that the overall 3-dimensional structure is sufficiently preserved that ability to bind to the Ang IV receptor at suitable affinity is retained. Using modern methods of peptide synthesis and combinatorial chemistry, it is possible to synthesize and test very large numbers of analogues within a short space of time, and such synthesis and screening is routinely carried out by pharmaceutical companies.

Considerable information is available regarding the structural features of Ang IV peptides which are necessary for high affinity, and these results may be used as guidelines for modification of the peptides of the invention. See for example Wright *et al*, 1995.

The person skilled in the art will appreciate that by modifying the sequence or by constructing a non-peptide analogue the activity of the compound of the invention can be very considerably modified. Not only can improvement in activity be obtained, it is also possible to obtain compounds which bind to the AngIV binding site in such a way that AngIV activity is inhibited. Such inhibitory compounds can have the ability to antagonize the activity of AngIV. The person skilled in the art will readily be able to synthesize modified peptides and peptide analogues and to test whether they have activity as AngIV agonists or antagonists, using methods well known in the art.

Thus according to a second aspect, the invention also provides compounds which are able to act as antagonists of the neuroactive peptides of the invention.

According to a third aspect, the invention provides a method of modulating motor neuron activity, cholinergic neuron activity, or neuronal development, comprising the step of administering an effective amount of a compound of the invention to a mammal in need of such treatment. This aspect of the invention specifically includes the use of decapeptide sequence referred to above in the method of the invention which relies on a previously unknown and unsuspected activity of the decapeptide.

Preferably the mammal is a human.

The AngIV agonist and antagonist compounds according to the invention are useful in the treatment of a variety of conditions, including but not limited to:

- Dementia, including Alzheimer's disease
- Other neurodegenerative disorders involving cholinergic pathways, motor pathways, or sensory pathways, such as motor neurone disease
- sensory and motor peripheral neuropathies
- brain or spinal cord injury due to trauma, hypoxia or vascular disease.

Detailed Description of the Invention

The invention will be now described in detail by way of reference only to the following non-limiting examples, and to the figures, in which

Figure 1 shows competition curves derived from prefrontal cortical sections incubated with $[^{125}I]$Ang IV in the presence of increasing concentrations of the following unlabelled ligands: ▲ Ang IV, □ Ang II, ■ Ang III, △ Ang II(1-7), ● losartan and ○ PD 123319. Values are the mean of four sections, each from two animals. B/Bo is the fraction of available receptors occupied;

Figure 2 shows the results of competition binding studies showing the inhibition of $[^{125}I]$Ang IV binding to E13 chicken chorioallantoic membranes with varying concentrations of unlabelled compounds: ▲ Ang IV, ◇ Nle^1-AIV, △ CGP 42112, □ Ang II, ▼ Nle^1-Y-I-amide, ■ WSU-4042, ■ [Sar^{1}Ile^8]Ang II, ● PD 123319 and ○ losartan. Values are expressed as a percentage of total binding, and are pooled from two experiments. B/Bo x 100=% of available receptors occupied;

Figure 3 summarizes competition binding studies showing the inhibition of ^{125}I[Sar^{1}Ile^8]Ang II binding to E13 chicken chorioallantoic membranes with varying concentrations of unlabelled compounds:

5 ■ [Sar^{1}Ile^8]Ang II, □ Ang II, △ CGP 42112, ◇ Nle^1-AIV, ▲ Ang IV, ○ osartan, ● PD 123319, ▆ WSU-4042 and ▼ Nle^1-Y-I-amide. Values are expressed as a percentage of total bnding, and are pooled from two experiments. B/Bo x 100 = % of available receptors occupied;

10 Figure 4 shows the effect of Ang IV on neurite outgrowth from E11 chicken sympathetic neurons. Values are expressed as a percentage of control levels, and are depicted as the mean±standard error of the mean (SEM). The results are pooled from 3 experiments, each with at least

15 40 neurite measurements. * indicates a significant difference from control values using Bonferroni's test;

Figure 5 shows the effect of 10 nM Ang IV on neurite outgrowth in the presence of 1 µM Nle^1-Y-I-amide, WSU-4042, Nle^1-AIV, [Sar^{1}Ile^8]Ang II, losartan, PD 123319

20 and CGP 42112. Values are expressed as a percentage of control levels, and are depicted as the mean±S.E.M. The results are pooled from 3 experiments, each with at least 40 neurite measurements. * indicates a significant difference from control values using Bonferroni's test;

25 Figure 6 shows the effect of 10 nM Ang II on neurite outgrowth in the presence of 1 µM Nle^1-Y-I-amide, WSU-4042, Nle^1-AIV, [Sar^{1}Ile^8]Ang II, losartan, PD 123319 and CGP 42112. Values are expressed as a percentage of control levels, and are depicted as the mean±S.E.M. The

30 results are pooled from 3 experiments, each with at least 40 neurite measurements. * indicates a significant difference from control values using Bonferroni's test;

Figure 7 illustrates the binding of ^{125}I-angiotensin IV to sheep spinal cord. The arrow indicates the site of damage to the spinal cord;

Figure 8 summarizes the results of competition binding studies showing the inhibition of [^{125}I]LVV-haemorphin-7 binding to sheep cerebellar cortical membranes with varying concentrations of unlabelled compounds: ▲ Ang IV, △ LVV-haemorphin-7, ■ Ang III, □ Ang II, ○ PD 123319, ● losartan, * naloxone and ∇ haloperidol. Values are the mean of three experiments. B/Bo x 100 = % of available receptors occupied;

Figure 9 summarizes the results of competition binding studies showing the inhibition of [^{125}I]AngIV binding to sheep cerebellar cortical membranes with varying concentrations of unlabelled compounds: ▲ AngIV, △ LVV-haemorphin-7, ■ AngIII, □ AngII, ○ PD 123319, ● losartan, * naloxone and ∇ haloperidol. Values are the mean of three experiments. B/Bo x 100 = % of available receptors occupied;

Figure 10 is a schematic diagram illustrating the position of the oligonucleotide probes used for cloning and PCR experiments. (A) schematic diagram of the β-globin precursor showing relevant position and direction of oligonucleotides used. The shaded region represents the LVV-haemorphin-7 sequence, which is given below. (B) sequences of the oligonucleotides used in this study;

Figure 11 illustrates the detection of β-globin mRNA by RT-PCR and Southern blotting in sheep cerebellar and cerebral cortices, heart and liver. Molecular weight markers are shown on the left;

Figure 12 shows the complete nucleotide sequence of Clone EX; and

Figure 13 shows the nucleotide sequence and derived amino acid sequence of the rat EX clone. The region of the potential LVV-haemorphin-7 is shown in bold.

The unlabelled ligands, Ang IV (Peninsula Laboratories, California USA), Ang II and the Ang II antagonist [Sar^1Ile^8]Ang II (Sigma, Missouri USA), the Ang II partial agonist CGP 42112 (Ciba-Geigy, Basle Switzerland), the Ang II AT_1 antagonist, losartan (Du Pont Merck Pharmaceutical Company, Delaware USA), the Ang II AT_2 antagonist, PD 123319 (Parke-Davis, Michigan USA-Ms. C.L.Germain), and the Ang IV analogues, WSU 4042, Nle^1-Y-I-amide and Nle^1-AIV (prepared as previously described by Sardinia et al, 1993), were used at final concentrations ranging from 10^{-9} to 10^{-4} M.

Example 1   Mapping of Angiotensin IV Receptors in
                   Monkey Brain

We mapped the distribution of the receptors for Ang IV (AT_4 receptors) in the *Macaca fascicularis* brain using *in vitro* receptor autoradiography in order to determine if the widespread and distinct distribution of the receptors that are found in the guinea pig brain is also found in primates. The binding sites were initially characterized pharmacologically in competition studies on prefrontal cortical brain sections. These results are summarized in Figure 1. Ang IV, Ang III and Ang II competed for [^{125}I]Ang IV binding with IC_{50}s of 5 nM, 80 nM and 730 nM respectively, while Ang II(1-7) was a weak competitor (IC_{50} of 24 mM). The AT_1 receptor antagonist, losartan (du Pont-Merck) and the AT_2 receptor antagonist, PD 123319 (Parke-Davis), were inactive, even at concentrations of 10 mM. These pharmacological properties are similar to those previously described for the AT_4

receptor in bovine adrenal and guinea pig septal membranes, confirming that we were mapping the distribution of the same receptor.

The distribution of the AT_4 receptor was
5 remarkable, in that its distribution extended throughout several neural systems. This is summarized in Table 1. The most striking finding was the localization of this receptor in motor nuclei and motor-associated regions. These included the ventral horn spinal motor neurons, all
10 cranial nerve motor nuclei including the oculomotor, trochlear, facial and hypoglossal nuclei, and the dorsal motor nucleus of the vagus. Receptors were also present in the vestibular, reticular and inferior olivary nuclei, the granular layer of the cerebellum, and the Betz cells of the
15 motor cortex. Moderate AT_4 receptor density was seen in all cerebellar nuclei, ventral thalamic nuclei and the substantia nigra pars compacta, with a lower receptor density being observed in the caudate nucleus and putamen. The localization of the AT_4 receptor in all levels of the
20 motor hierarchy in the central nervous system implies an important role for the binding site in motor activity.

Table 1

Localization and Quantitation of the
AT_4 Receptor in the *Macaca fascicularis* Brain

Region	AT_4 receptor density dpm/mm2 (mean ± SD)
Caudate nucleus	48 ± 2
Vertical limb of the diagonal band*	86 ± 3
Basal nucleus of Meynert*	81 ± 5
Granular layer of the dentate gyrus	117 ± 11
CA1	45 ± 4
CA3	41 ± 3
Supraoptic retrochiasmatic nucleus*	93 ± 7
Ventral posterior lateral/medial nuclei	35 ± 2
Red nucleus*	44 ± 2
Oculomotor nucleus*	44 ± 1
Pontine nuclei	50 ± 2
Lateral geniculate	52 ± 2
Mo5*	84 ± 3
Facial nucleus*	90 ± 4
Hypoglossal nucleus*	93 ± 8
Inferior olive	76 ± 10
Granular layer of the cerebellum	126 ± 10
Molecular layer of the cerebellum	47 ± 6

Values are the mean of four sections from one animal and are representative of the relative densities of AT_4 receptors. * Values are determined from the overall area and not from individual cell bodies which exhibit higher binding.

In addition to the somatic motor nuclei and autonomic preganglionic motor nuclei, abundant AT_4 receptors were also found in other cholinergic systems and their projections, including the nucleus basalis of Meynert, vertical limb of the diagonal band and the hippocampus. Apart from being a neurotransmitter in motor neurons, acetylcholine is also implicated in cognition, since anti-cholinergic drugs induce memory disorders and confusion; in Alzheimers's disease, neuronal loss occurs in the cholinergic-rich basal nucleus of Meynert. Ang IV has been shown by two independent studies to facilitate memory retrieval in passive and conditioned avoidance tests (Braszko et al, 1988; Wright et al, 1993), and, when administered intracerebroventricularly, induces c-fos expression in the hippocampus (Roberts et al, 1995). Together with the presence of high densities of AT_4 receptors in this region, these observations suggest that Ang IV may play an important role in the modulation of cognitive function.

AT_4 receptors were also observed in sensory regions, with moderate levels in spinal trigeminal, gracile, cuneate and thalamic ventral posterior nuclei, and in the somatosensory cortex. While receptor density was low in sensory neurons when compared with that observed in motor and cognitive areas, the AT_4 receptor was located throughout all sensory-associated areas, including the lamina II of the spinal cord, gracile, cuneate and spinal trigeminal nuclei, ventral posterior thalamic and lateral geniculate nuclei and the sensory cortex, suggesting a substantial involvement with sensory activity. This distribution pattern has also been observed in the guinea pig and sheep brain. As shown in Example 2, abundant AT_4 receptors were also observed in sheep dorsal root ganglia.

Example 2   Mapping of Angiotensin IV Receptors in
                   Sheep Spinal Cord

We extended the localization of the AT_4 receptors
to the sheep spinal cord, to investigate if the strong
presence of the AT_4 receptors in supraspinal motor and
sensory regions persists in the spinal cord.

When the binding characteristics of $[^{125}I]$Ang IV
were assessed in the eighth cervical segment (C8) of the
sheep spinal cord, we found that the affinities of the
different unlabelled ligand in competing for the binding
were similar to those observed for the monkey brain.

In the sheep spinal cord, high densities of AT_4
receptors were found in lamina IX in the ventral horns of
all segments examined. At a cellular level, the binding
was found overlying the cytoplasm of lateral and medial
motor neurons and in their processes, but binding was
absent from the cell nuclei. Whilst a clearly defined
function of the Ang IV binding site is yet to be
determined, the association with motor activity is
strengthened in view of its abundant localization in the
motor neurones in the ventral horn of the spinal cord, in
addition to its strong presence in supraspinal motor areas.

High densities of AT_4 receptors were also found
in the lateral tip of lamina VII of all thoracic segments
and lumbar segments L1 to L4, which corresponded with
sympathetic preganglionic neurons in the intermediolateral
cell column. However, binding was absent from L5 and L6
and from the sacral segments S1 and S2.

In the dorsal root ganglia associated with all
spinal segments, high densities of AT_4 receptors were found
in the cytoplasm of small and large cell bodies of the
sensory neurons, but not in the satellite cells, nor in the
endoganglionic connective tissue. In laminae I and II, the

terminal fields of the dorsal root ganglia sensory afferents, only a low abundance of the receptor was noted in lamina II. Despite the low levels of AT_4 receptors in lamina II, their high abundance in the dorsal root ganglia

5 and their consistent but low levels in most supraspinal sensory areas suggest that AT_4 receptors may still play a role in the processing of sensory information.

Low levels of the AT4 receptors were also found in the blood vessels which extended radially to the pial

10 surface, in the blood vessels of the anterior and posterior fissures, and in the ependyma of the central canal. Ang IV has been reported to induce an endothelium-dependent dilation of rabbit pial arterioles, and in rats Ang IV reverses acute cerebral blood flow reduction after

15 experimental subarachnoid haemorrhage.

Our localization studies suggest that AT_4 receptors are quite distinct from the known angiotensin receptors -the AT_{1a} , AT_{1b} and AT_2 receptors - in terms of their pharmacological specificity and their pattern of

20 distribution in the brain and spinal cord. Furthermore, the pattern of distribution of the AT_4 receptors suggests that they may be involved in the function of neurones involved in motor function, sensory function and cholinergic systems, including cognition.

25

Example 3   Characterization of Embryonic Chicken Ang
                   IV and Ang II Binding Sites

In order to characterize the pharmacology of the embryonic chicken AT_4 and Ang II receptors, chorioallantoic

30 membranes (CAM) from embryonic day 13 (E13) chickens were used. The membranes were removed and frozen in isopentane cooled to -40°C.

a)   Characterization of the embryonic chicken Ang IV
          binding site

CAM were homogenized in 30 ml of a hypotonic
buffer (50 mM Tris, pH 7.4, 5 mM EDTA) and then centrifuged
for 10 min at 500 g and 4°C. The supernatant fraction was
removed and centrifuged for 20 min at 40,000 g and 4°C.
The resulting pellet was rehomogenized in 2 ml of hypotonic
buffer, and the final volume of the homogenate was adjusted
to give a protein concentration of 10 mg/ml, as determined
by the Biorad protein assay. The binding assay contained
CAM (100 µg of protein), 0.14 µCi of [^{125}I]Ang IV
(approximately 260 pM), and competing ligand, in a total
volume of 270 µl in a 50 mM Tris buffer, pH 7.4, containing
150 mM NaCl, 5 mM EDTA, 100 µM phenylmethylsulfonyl
fluoride, 20 µM bestatin and 0.1% (w/v) bovine serum
albumin. The binding system was incubated at 37°C for 2 h.

b)   Characterization of the embryonic chicken Ang II
          binding site

CAM were prepared as described above with the
following exceptions. The isotonic buffer contained 50 mM
Tris, pH 7.4 and 6.5 mM MgCl$_2$ and the hypotonic buffer
contained 50 mM Tris, pH 7.4, 6.5 mM MgCl$_2$, 125 mM NaCl and
0.2% (w/v) bovine serum albumin. In addition, the
peptidase inhibitors, leupeptin, lisinopril,
phosphoramidon, Plummer's inhibitor and bestatin, each used
at a 1 µM concentration and 1 mM benzamidine and 2.5 mM
phenanthroline, were included in both buffers.

In binding competition studies on E13 chicken
CAM, [^{125}I]Ang IV binding was strongly inhibited by Ang IV
and Nle^1-AIV (IC$_{50}$s of 18 and 43 nM respectively), whereas

WSU-4042, Nle^1-Y-I-amide and Ang II were weaker competitors with IC$_{50}$s of 5, 2.2 and 0.65 μM respectively, and losartan and PD 123319, were inactive at concentrations up to 10 μM. [Sar^{1}Ile^8]Ang II and CGP 42112 were effective at only competing for 50% of the sites, and then only at concentrations of 10 and 0.5 μM respectively. These results are summarized in Figure 2.

In studies of ^{125}I[Sar^{1}Ile^8]Ang II binding to CAM, Ang II, [Sar^{1}Ile^8]Ang II and CGP 42112 competed for binding with IC$_{50}$s of 100, 13 and 180 nM respectively, whilst Ang IV, Nle^1-AIV and losartan were very weak competitors (IC$_{50}$s of 50, 8 and 100 μM respectively). PD 123319, WSU-4042 and Nle^1-Y-I-amide exhibited IC$_{50}$s greater than 100 μM. These results are shown in Figure 3.

Example 4   Effects of Ang IV on Neurite Outgrowth

The wide distribution of the AT$_4$ receptors in motor, sensory and cholinergic regions suggests important roles for this peptide in the central nervous system. However, a physiological action of Ang IV in neurons has yet to be clearly defined. Numerous neurotransmitters and neuropeptides have been associated with the regulation of neuronal development. For instance, acetylcholine inhibits neurite outgrowth from embryonic chicken ciliary ganglion cells, sympathetic neurons, and rat hippocampal neurons. Conversely, vasoactive intestinal peptide stimulates superior cervical ganglion branching and somatostatin increases neuronal sprouting from *Helisoma* buccal ganglion neurons.

We determined whether Ang IV has a trophic role in the central nervous system by examining its effects on

neurite outgrowth from cultured embryonic chicken
sympathetic neurons.

Sympathetic ganglia from E11 chickens were
dissociated using trypsin/Versene, and were cultured in 24
well plates in DMEM and Ham's F12 medium which contained 1%
(v/v) insulin-transferrin-selenium-X growth supplement
(Gibco BRL, Maryland USA), 100 mM putrescine, 1.67 mg/ml
prostaglandin F2α, 6.67 ng/ml progesterone, and 5 ng/ml
nerve growth factor (Sigma, Missouri USA). Neurons were
allowed to adhere to the wells (approximately 2 h) before
being given a 24 h treatment of peptides and/or their
antagonists. Peptides and antagonists used were added to
the cultures 0.5 h prior to either Ang IV or Ang II
addition. Ang IV dose response curves were performed over
the concentration range 10^{-11} to 10^{-5} M. Culture dishes
were coated with 0.1 mg/ml polylysine and then given three
washes with phosphate-buffered saline (PBS) before being
coated with 10 μg/ml laminin. Wells were washed with PBS
before being used for culture.

At the conclusion of the experiment, the culture
medium was removed from the wells, the neurons were fixed
with 2.5% glutaraldehyde in PBS for 20 min and examined
under a phase-contrast microscope, attached to an MD30 Plus
image analysis software (Adelaide, Australia). The length
of neurites (longer than 50 μm) of every neuron examined
was measured. A minimum of forty neurite measurements was
taken per treatment group, and each experimental treatment
was performed at least in triplicate.

At the conclusion of the experiment, the
viability of the cells were confirmed by exclusion of 0.1%
aniline blue.

In cultures of embryonic (E11) chicken
sympathetic neurons, Ang IV inhibited neurite outgrowth in

a dose-dependent manner, with a threshold at 10^{-11} M, half maximal inhibition at 10^{-10} M and a maximal effect at 10^{-9} M. Between 10^{-9} to 10^{-5} M, outgrowth was maximally inhibited ($P<0.05$). These results are shown in Figure 4.

5   At 10^{-8} M Ang IV, the inhibition of neurite outgrowth was totally reversed by 1 μM of the Ang IV analogues WSU-4042, Nle^1-Y-I-amide, and Nle^1-AIV. The effects of the analogues alone were not statistically different from control values. In contrast to the Ang IV analogues, the Ang II antagonist,

10   $[Sar^1Ile^8]$Ang II, the AT_1 and AT_2 antagonists, losartan and PD 123319, and the Ang II partial agonist, CGP 42112, had no effect on the Ang IV response, as shown in Figure 5.

At 10^{-8} M Ang II, neurite outgrowth was inhibited by 25%, which was highly significant. The Ang IV analogues

15   completely reversed this effect, whilst the Ang II antagonists $[Sar^1Ile^8]$Ang II, losartan, PD 123319, and CGP 42112 were ineffective. This is illustrated in Figure 6.

These studies suggest that the inhibition of

20   neurite outgrowth by both peptides is mediated by the AT_4 receptors, and supports a role for angiotensin IV in neurite modelling.

Example 5   Effect of Angiotensin IV on Spinal Cord

25   Damage

Glial fibrillary acid protein (GFAP)-positive astrocytes are involved with modelling neurite formation after damage to the spinal cord (Bovolenta et al, 1992). Injury-evoked plasticity is a similar situation to that

30   observed in the developing embryo (Schwartz, 1992). In light of our findings on the ability of spinal cord tissue to bind Ang IV (Example 2), we tested the effect of spinal cord injury on Ang IV binding. Surprisingly, we found a

marked elevation of [^{125}I]Ang IV binding in damaged spinal
cord sections. This is illustrated in Figure 7.

These results suggest that the AT$_4$ receptor may
be a suitable target for alleviation of the effects of
5 spinal cord injury.

Example 6   Purification of an Endogenous Brain Peptide
                Which Binds to the AT$_4$ Receptor

The level of Ang IV in the brain is very low to
10 undetectable (DJ Campbell, personal communication). The
widespread and characteristic distribution of AT$_4$ receptors
in the central nervous system suggests that there may be an
as yet unidentified peptide ligand for this receptor. We
therefore undertook a search for such a ligand, using
15 conventional protein chemistry purification techniques
together with an AT$_4$ receptor assay system in order to
detect and monitor substance(s) in extracts of sheep brain
which compete for [^{125}I]Ang IV binding in this system.

20 a) ^{125}AT$_4$ Receptor Binding Assay

The binding of ^{125}I-Ang IV to bovine adrenal
membranes was used as an assay system to screen for AT$_4$
receptor binding activity in sheep cerebral cortex
fractions. Bovine adrenal glands obtained from the
25 abbatoir were diced into 1 mm x 1 mm blocks, homogenized in
3 ml of a hypotonic buffer (50 mM Tris, 5 mM EDTA, pH 7.4)
and then centrifuged for 10 min at 500 g. The supernatant
was removed and centrifuged for 20 min at 40,000 g, and the
resulting pellet was rehomogenized in 2 ml of hypotonic
30 buffer. Binding assay samples contained bovine adrenal
(56 mg of protein as determined by the Biorad protein
assay), 0.14 μCi of [^{125}I]Ang IV (approximately 260 pM),
and 10 μl of test sample, in a total volume of 270 ml in

50 mM Tris buffer, pH 7.4, containing 150 mM sodium chloride, 5 mM EDTA, 100 μM phenylmethylsulfonyl fluoride, 20 μM bestatin, and 0.1% (w/v) bovine serum albumin. The relative potency of the fractions in competing for

5 ^{125}I-Ang IV binding was determined from a standard curve in which known amounts of unlabelled Ang IV were added (10^{-10} to 10^{-6} M). Fractions from each purification step were assayed for their ability to compete for [^{125}I]Ang IV binding, with those exhibiting the highest activity

10 undergoing the next purification step.

b)   Purification Procedure

          Sheep cerebral cortex was homogenized in 2 M acetic acid, (2 ml/g tissue), centrifuged, and the

15 supernatant decanted. A preliminary purification of the extract was performed using a column of preparative C18 material (55-105 mm, Waters). The C18 eluent was lyophilized, reconstituted, and subjected to a series of chromatographic steps, in which fractions were assayed for

20 Ang IV displacement activity. In brief, the chromatographic steps were: three successive reversed-phase HPLC steps, using columns of varying pore size (Deltapak C18, 300°A, and Novapak C18) as well as changing ion-pairing agents, solvents and gradient elution conditions;

25 this was followed by anion exchange, then cation exchange, with final purification on a microbore LC C8 column. The purified active peptide was sequenced using an Applied Biosystems Model 470 A Protein Sequencer with an on-line Model 120A PTH Analyzer.

30   The sheep cerebral cortex yielded 1.9 nmoles of AT$_4$ receptor binding activity per gram of wet weight after the first C18 Deltapak column. Following the third Poly LC column (55°C), Ang IV activity coeluted with the major UV

absorption peak, and the following peptide sequence was obtained from this peak:

Leu-Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe.

A search of protein database records revealed
that this sequence corresponded to the amino acid sequence 32-41 of the human β, δ, γ and ϵ globin chains and is known as LVV Haemorphin 7.

LVV-haemorphin 7 is a 10 amino acid peptide found in the brain, pituitary, hypothalamus and bone marrow which binds with high affinity to the angiotensin AT_4 receptor. The sheep peptide sequence is identical to amino acids 31-40 of the sheep β_A, β_B, β_C, and ϵ globin precursors (Garner and Lingrel, 1989; Saban and King, 1994), and this sequence is conserved in many species, including human (see for example Karelin et al, 1994). In humans, there are 6 β-globin-like genes ϵ, γ^A, γ^G, δ, β and a pseudogene $\psi\beta$, clustered on chromosome 11, and all encode the LVV-haemorphin 7 sequence (Karlsson and Nienhuis, 1985). This sequence is not present in any of the α globin family of genes. LVV-haemorphin-7 and some shorter sequences within this peptide have opioid activity, and it appears that the sequence VVYP is required for this activity (Karellin et al, 1994).

Example 7   Properties of Synthetic LVV-Haemorphin 7

A decapeptide with the sequence isolated above was synthesized under contract by Chiron Mimotopes, and its biochemical and pharmacological properties were characterized as follows:

a)   HPLC

A preliminary hplc run indicated that the synthetic peptide did not coelute with the fraction that

was sequenced. It appeared that the fraction might have been degraded due to prolonged storage at 4°C. Mass spectrometry analysis was carried out in order to determine whether this was the case. The data obtained from mass

5 spectrometry analysis of the two active peaks produced following prolonged storage of the original purified material were indeed consistent with degradation. The early eluting peak gave a mass corresponding exactly to the loss of the phenylalanine residue from the carboxy terminus,

10 whereas the second active peak gave a mass corresponding exactly to the loss of the amino terminal leucine residue. Furthermore, these data (given that all the mass readings were unambiguous) strongly suggest that the active peptide is not post-translationally modified, either in the peptide

15 core or at the amino or carboxyl terminus.

b)   Ligand Binding Studies

The pharmacological properties of the decapeptide LVV-haemorphin 7 in competing for the binding of ^{125}I-Ang IV

20 in bovine adrenal membrane and sheep cerebellar cortical membranes were determined. Both LVV-haemorphin-7 and Ang IV were radioiodinated using chloramine T, and separated on a C18 Sep-pak column using 0.5% trifluoroacetic acid in a 20-80% methanol gradient.

25   Bovine adrenal membranes or sheep cerebellar cortical membranes were homogenized in 30 ml of a hypotonic buffer (50 mM Tris, 5 mM EDTA, pH 7.4), and then centrifuged for 10 min at 500 g. The supernatant was removed and centrifuged for 20 min at 40,000 g, and the

30 resulting pellet was rehomogenized in 2 ml of hypotonic buffer. Binding assays contained:

bovine adrenal (56 μg of protein) or sheep
cerebellar membranes (26 μg of protein), as determined by
the Biorad protein assay (Bradford, 1976);

0.14 μCi of [^{125}I]Ang IV (approximately 260 pM),
5   or 0.11 μCi of [^{125}I]LVV-haemorphin-7 (approximately
200 pM), and

competing ligand,

in a total volume of 270 ml in 50 mM Tris buffer,
pH 7.4, containing 150 mM sodium chloride, 5 mM EDTA, 10 mM
10   phenylmethylsulfonyl fluoride, 20 μM bestatin and
0.1% (w/v) bovine serum albumin.

The assay was incubated at 37°C for 2 h.

In the bovine adrenal membranes, a range of
concentrations of unlabelled LVV haemorphin 7 or Ang IV was
15   added to the assay system in order to determine the
relative potencies of the two peptides in this
radioreceptor assay system. Both Ang IV and LVV haemorphin
7 displayed comparable affinities in competing for the ^{125}I-
Ang IV binding (approx. 1-5 nM), with Ang IV exhibiting
20   slightly higher affinity.

For competition studies in sheep cerebellar
cortical membranes, dilutions of the unlabelled ligands,
LVV-haemorphin-7, Ang IV, Ang II, Ang III and the non-
specific opioid antagonist, naloxone, the Ang II AT$_1$
25   antagonist, losartan, the Ang II AT$_2$ antagonist, PD 123319,
and the sigma opioid and dopamine D$_2$ antagonist,
haloperidol, were used at concentrations ranging from 10^{-13}
to 10^{-4} M. Quantitation of receptor binding was calculated
as the mean of two experiments.

30   In these studies, ^{125}I-LVV-haemorphin-7 binding
to sheep cerebellar cortical membranes was competed for by
LVV-haemorphin-7, Ang IV, Ang III, and Ang II (IC$_{50}$s of

5.6 nM, 1 nM, 77 nM, and 1.6 μM respectively). PD 123319 was a weak competitor (IC_{50} of 46 μM), whilst losartan, naloxone and haloperidol were ineffective (IC_{50} greater than 100 mM). These results are illustrated in Figure 8.

5   Similarly, [^{125}I]Ang IV binding to cerebellar membranes was competed for by Ang IV, LVV-haemorphin-7, Ang III, and Ang II with IC_{50}s of 1.13 nM, 2 nM, 6.9 nM and 2 μM respectively, whilst PD 123319, losartan, naloxone and haloperidol were inactive at 10 μM. These results are

10   illustrated in Figure 9.

c)   <u>Binding of ^{125}I-LVV Haemorphin 7 to Sheep Brain</u>
      Sheep hindbrain sections were used to compare the distribution of ^{125}I-LVV-haemorphin-7 binding and AT_4

15   receptor sites. Sections at 10 mm thickness were equilibrated to 22°C (30 min), and then preincubated for 30 min in an isotonic buffer containing 50 mM Tris, 150 mM sodium chloride, 5 mM EDTA, 100 μM phenylmethylsulfonyl fluoride, 20 μM bestatin and 0.1% bovine serum albumin,

20   pH 7.4, before a further 2 h incubation in the same buffer containing 2.84 μCi of [^{125}I]LVV-haemorphin-7 or [^{125}I]Ang IV (approximately 140 pM). The binding of the radioligands was cross-displaced with either 1 μM unlabelled LVV-haemorphin-7 or Ang IV. After incubation,

25   the sections were given three 2 min washes in buffer at 4°C, and exposed to X-ray film for 14 to 28 d.
      [^{125}I]LVV-haemorphin-7 and [^{125}I]Ang IV exhibited an identical binding pattern in the sheep hindbrain. Binding was localized to the motor-associated areas, the

30   granular layer of the cerebellum, the inferior olive, hypoglossal and lateral reticular nuclei, to the autonomic regions, the dorsal motor nucleus of the vagus and the

nucleus ambiguus, and to the sensory regions, the external cuneate and spinal trigeminal nuclei. The binding of both radioligands was displaced by a 1 μM concentration of either unlabelled Ang IV or LVV haemorphin 7, indicating that not only are the two binding sites distributed in the same brain regions, but that the two radioligands are actually binding to the same sites.

Example 8   Isolation of Potential LVV-Haemorphin-7 Precursor Clones

It is not known whether LVV-haemorphin-7 is synthesized in the brain, or whether it is derived from the breakdown of haemoglobin. Demonstration of LVV-haemorphin-7 precursor mRNA in the brain would provide evidence for the former. Possible methods to demonstrate that LVV-haemorphin-7 precursor mRNA is present in the brain include:

(a)   isolation of specific cDNA clones from a brain cDNA library;

(b)   detection of the mRNA in the brain by PCR;

(c)   detection of LVV-haemorphin 7 precursor mRNA by *in situ* hybridization histochemistry; and

(d)   demonstration of the mRNA in brain specific cell cultures.

It has previously been reported that α- and β-globin mRNAs are expressed in mouse brain, as demonstrated by Northern analysis (Ohyagi,Y., *et al*, 1994).

Each of these approaches has specific advantages. *In situ* hybridization histochemistry and detection of the mRNA in brain specific cell cultures would provide evidence for synthesis in the brain. Isolation of clones and the reverse transcription polymerase chain reaction (RT-PCR) detection of mRNA would show the presence of mRNA in the brain, but contamination by reticulocytes cannot be

excluded. However, isolation of cDNA clones provides considerable information about the structure of the precursor. The precursor of LVV-haemorphin-7 may be a member of the β-globin family, eg β^A, ε , etc, or an
5   alternatively spliced globin, or it may be a previously unknown non-globin peptide.

To isolate potential clones that code for the precursor of the LVV-haemorphin-7 peptide, we have screened a rat brain cDNA library using an oligonucleotide based on
10   the LVV-haemorphin-7 sequence.

Oligonucleotide Design

A number of oligonucleotides have been designed, as illustrated in Figure 10. Oligonucleotide H170 was
15   designed to correspond to the region of the sheep β-globin gene encoding the LVV-haemorphin-7 sequence. This probe was used for screening the library, and also as the sense oligonucleotide in PCR. Oligonucleotide H173 was designed as the antisense primer for use in PCR. PCR with H170/H173
20   spans intron 2, and will generate a 255 bp fragment with cDNA as the template and a 1098 bp fragment with genomic DNA. Oligonucleotide H172 can be used as an internal probe for H170/H172 PCR products. Oligonucleotide H171 is an antisense probe corresponding to the region of the sheep β-
25   globin gene encoding the LVV-haemorphin 7 sequence, and was used for in situ hybridization histochemistry, with H170 as the matching sense control oligonucleotide.

Detection of β-Globin Like Sequences in Brain by Polymerase
30   Chain Reaction (PCR)

RNA was isolated from sheep cerebellar and cerebral cortices, heart and liver. The RNA (20 µg) was reverse transcribed in a 25 µl reaction containing 100mM

KCl, 50mM Tris-HCl (pH 8.4), 6 mM MgCl$_2$, 10 mM

dithiothreitol, 500µM dNTPs (Progen), 12µg/ml random

hexamers (Boehringer Mannheim), 40 units RNasin (Progen),

and AMV reverse transcriptase (Boehringer Mannheim, 25

5   units) at 42°C for 1 h. An aliquot of the reverse

transcription reaction (10%) was used in the polymerase

chain reaction. The primers used for amplification of the

β-globin mRNA were sense H170 and antisense H173 (see

Figure 10). PCR was performed in a reaction containing:

10   10mM Tris-HCl (pH 8.3), 50mM KCl, 400µM dNTPs, Taq

Polymerase (Bresatec, 2.5 units), 3µM MgCl$_2$,and each primer

at 400nM. Denaturation, annealing and extension were

carried out at 94°C, 60°C and 72°C for 1 min each for 40

cycles, followed by a final extension at 72°C for 10 min.

15   The PCR products were separated on an agarose

gel, transferred to Hybond N+, and Southern analysis using

an internal oligonucleotide (H172) was performed to confirm

that the products were derived from globin precursors.

Specific bands of the expected size of 255 bp were detected

20   in all four tissues examined, as shown in Figure 11.

Screening a Rat cDNA Library for β-Globin Like Sequences

An oligonucleotide corresponding to the

nucleotide sequence of the LVV-haemorphin-7 region of the

25   sheep β-globin (H170) was used to screen a rat brain cDNA

library (Stratagene Cat No: 936515, Sprague-Dawley, whole

brain). Approximately 8 x 10^5 clones were plated, and

plaque lifts taken using standard methods (eg Maniatis et

al: Molecular Cloning). The filters were prehybridzed in

30   Rapid-Hyb (Amersham) for 1 hr at 42°C, then the 5' end

labelled H170 was added for 2 hr. The filters were then

washed 3 times at 42°C in 2xSSC/0.1% SDS. The filters were

autoradiographed for 4 days using Biomax film and an

intensifying screen. A total of 24 putative positives was isolated. The positives were eluted in PSB.

The positives were then further characterized using a PCR based method. PCR was performed using oligonucleotide H170 as the 5' primer and H173 as the 3' primer. A PCR product derived from H170/H173 will span an intron in the sheep β-globin gene, and will generate a 255 bp fragment.

An aliquot of the eluted λ clone was boiled for 5 min, then chilled on ice. This was used as template DNA in a PCR reaction containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 400 μM dNTPs, Taq Polymerase (Bresatec, 2.5 units), 3 μM MgCl$_2$, and each primer at 400 nM. Denaturation, annealing and extension were carried out 94°C, 60°C and 72°C for 1 min each for 30 cycles, followed by a final extension at 72°C for 10 min. PCR products were analysed by electrophoresis on a 1.4% agarose gel.

The H170 positive/PCR negative clones were stored for further characterization. It is considered that they may be either non-globin precursors, alternatively spliced precursors or fragments of globin clones.

Sequencing Rat β-globin Clones

The 6 positives selected by PCR were plaque purified, and subjected to plasmid excision according to the manufacturers instructions. The insert sizes were determined by separate restriction mapping with the enzymes EcoRI and PvuII. Clones EX, FX, LX, RX and TX contain inserts of approx 500 bp. Clone DX was the longest, and contained an insert of approximately 2500bp. Southern analysis of the clones using an internal oligonucleotide (H172) confirmed that these clones were derived from globin precursors.

These plasmids were sequenced using the Pharmacia
T7 sequencing kit.   Sequencing of clones EX, FX and LX,
using the universal primer, showed sequence homology to the
3' untranslated region of β-globin.   Clones RX and TX when

5   sequenced with the universal primer, and clone DX when
sequenced with the reverse primer, showed sequence homology
to the 5' end of the β-globin gene, including the
initiation codon ATG.

Clone DX was subjected to nested deletion

10   analysis to generate more templates for sequencing.   This
clone contained the β-globin sequence, and approximately
1.8 kb of sequence which was not homologous to the globin
cluster, and may be the result of two inserts in the one
clone.

15   Complete sequencing of clone EX showed that the
clone was identical to rat β^A-globin (Genbank accession No:
X16417), as shown in Figure 12.   Figure 13 shows the
nucleotide sequence and derived amino acid sequence of
clone EX, indicating the putative LVV-haemorphin-7 region.

20

Example 9   *In situ Hybridization Histochemistry*
The distribution of mRNA encoding LVV haemorphin
7 and its precursor peptide is being investigated using a
range of oligonucleotides for the different regions of the

25   β-globin gene, including the C-terminal of exon 2 (H172 of
Figure 13) and the N-terminal regions of exon 3 (H173).
Both sense and antisense (initially H172, H173)
oligonucleotides were 3' end labelled with a ^{35}S-dATP using
terminal d-transferase and purified on a Nensorb column.

30   Sheep brain sections were then hybridized with
7.5×10^5 cpm of labelled probe in a 75 μl total volume of
50% formamide, 4xSSC, 1xDenhardt's solution, 2% sarcosyl,
20 mM Na_2PO_4 buffer (pH 7), 10% dextran sulphate, 50 μg/ml

herring sperm DNA and 0.2 M dithiothreitol. After a 16 h
hybridization period, the sections were washed four times
in 1 x SSC, rinsed in distilled water and dehydrated
through increasing ethanol and exposed to Hyperfilm β-max.

5   Preliminary experiments using oligonucleotides H172 and
H173 detected β-globin mRNA in the inferior colliculus and
nucleus of the spinal trigeminal. Further in situ
hybridization histochemical studies involve the use of
additional antisense and sense synthetic oligonucleotides

10   from different regions of the β-globin sequence to confirm
our finding of β-globin mRNA in brain nuclei. The
distribution of β-globin mRNA is then compared to our
autoradiographic localization of the AT$_4$ receptors in order
further to lucidate roles for this novel peptide system.

15

Example 10   Radioimmunoassay and Immunohistochemical
                                 Detection of LVV Haemorphin 7

        Two sheep were immunized with the LVV haemorphin
7 sequence conjugated to diphtheria toxoid and both

20   antisera and affinity purified antisera with adequate titre
to set up radioimmunoassays for LVV haemorphin 7 have been
obtained. The radioimmunoassay, which is of conventional
type, is used to determine the concentration of LVV
haemorphin 7 in different tissues or in specific regions

25   within a tissue, in order to provide us with further
information as to other possible physiological actions of
the decapeptide.

        The antisera are also used immunohistochemically
to determine the tissue distribution of LVV haemorphin 7,

30   particularly in the brain. Guinea pigs are perfused
intracardially with 4% paraformaldehyde in phosphate-
buffered saline solution, the tissues dissected out and
immersed in a 20% sucrose solution overnight. The tissues

are then frozen, 5-10 micron sections cut, and endogenous
peroxidase blocked by a 30 min incubation in 0.5% hydrogen
peroxidase in methanol prior to an overnight incubation
with the primary antibody in phosphate-buffered saline

5   containing 3% normal goat serum. After a few washes in
phosphate buffered saline, the sections are incubated with
the secondary anti-sheep antibody, and detected using the
streptavidin-biotin/horseradish peroxidase complex system
(Vectastain). The detection of LVV haemorphin 7 in neurones

10   provides further support that the decapeptide is
synthesized within neurones, and thereby may function as a
neuropeptide, since we have already shown that its receptor
occurs in neurones. Immunohistochemistry is also performed
at the electron microscopic level in order to evaluate the

15   subcellular distribution of the peptide, in particular
whether it occurs in intracellular storage granules.

       The radioimmunoassay for LVV haemorphin 7 is also
employed to investigate the secretion of the peptide from
neural tissue. Slices prepared from brain regions found to

20   be rich in LVV haemorphin 7 immunoreactivity are incubated
in Krebs Ringer Bicarbonate buffer at 37°C, and the effects
of depolarization by high K$^+$ medium and various
secretagogues are evaluated to test whether the peptide is
secreted from neurones. Similar experiments are carried

25   out on cultured neuronal cell lines which are found to
contain the peptide. Radioimmunoassays of body fluids
including plasma and cerebrospinal fluid are used to
determine levels of the peptide in these fluids under
normal and pathological conditions.

30   In addition, the subcellular distribution of the
peptide is evaluated by radioimmunassay of subcellular
fractions from nervous tissues, including synaptosomes, in

order to evaluate if the peptide is stored in subcellular granules, as occurs for other secreted neuropeptides.

We have mapped the distribution of AT_4 receptors in the brain of *Macaca fascicularis* and sheep spinal cord.
5 The receptor has a striking and unique distribution, including motor- and sensory-associated regions and pathways and cholinergic cell bodies, including all motor nuclei in the brain stem and spinal cord. We have demonstrated that AngIV inhibits neurite outgrowth in
10 cultured embryonic chicken neurones, and that this peptide may therefore have a role in growth and development of the central and peripheral nervous systems.

We have purified an endogenous brain peptide which binds to the AT_4 receptor with high affinity. This
15 decapeptide is 100% identical to the internal amino-acid sequence 31-40 of sheep β-globin. The presence of this β-globin-like sequence was demonstrated in sheep brain and other tissues using PCR. Screening of a rat brain cDNA library led to the isolation of a clone identical in
20 sequence to rat $β^A$-globin.

We have demonstrated the presence of β–globin mRNA in brain tissue and isolated a β-globin cDNA clone from a rat brain library. These data suggest that LVV-haemorphin-7 is derived from β–globin precursors
25 synthesized in the brain, although contamination by reticulocytes cannot be excluded. All of the cDNA clones sequenced correspond to the sequence encoding rat $β^A$-globin. The rat LVV-haemorphin-7 peptide sequence has a conservative substitution at position 10, with a tyrosine
30 replacing a phenylalanine.

It therefore appears that a peptide corresponding to the sequence of the bovine LVV-haemorphin-7 exists in

brain, and is derived from β-globin as precursor. The peptide is almost certainly the endogenous ligand for abundant brain AT_4 receptors, and may therefore exert a range of action on defined motor sensory and cholinergic

5   neurones. In a wider context, our findings suggest that β-globin may be a precursor of a range of neuroactive peptides generated in the central nervous system by specific cleavage enzymes to interact with a range of receptors.

10

It will be apparent to the person skilled in the art that while the invention has been described in some detail for the purposes of clarity and understanding, various modifications and alterations to the embodiments

15   and methods described herein may be made without departing from the scope of the inventive concept disclosed in this invention.

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20   following pages, and are incorporated by this reference.

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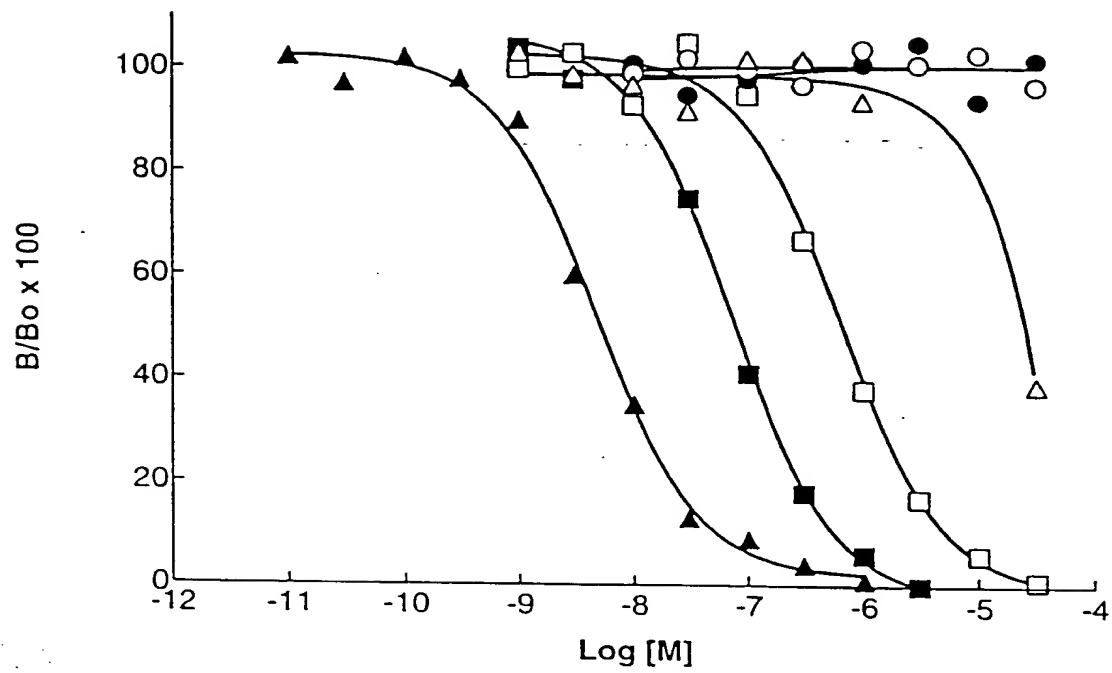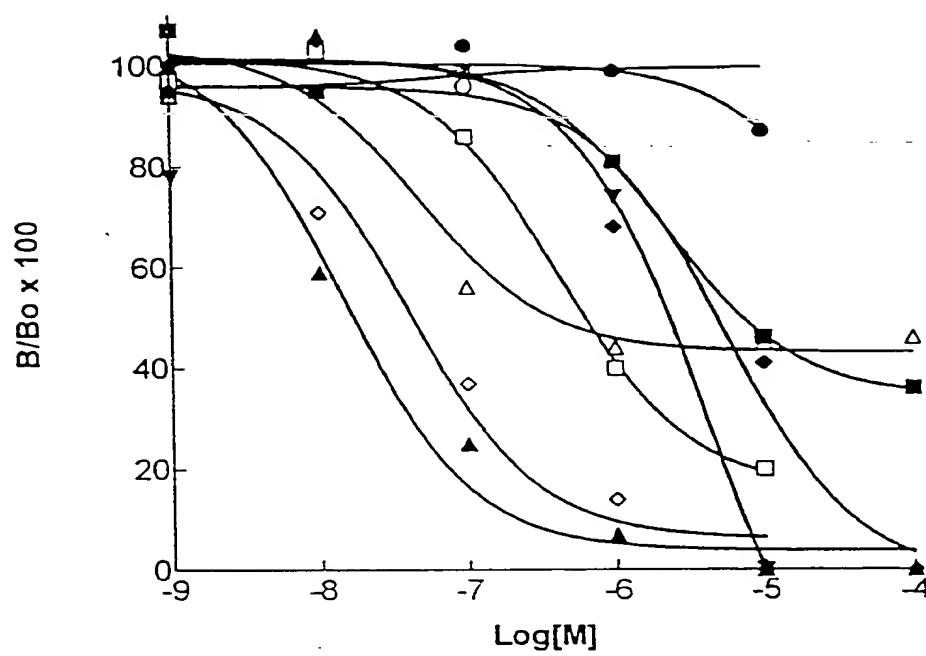16. Wright, J.W., Krebs, L.T., Stubb, J.W. and Harding, J.W.
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FIGURE 1

FIGURE 2

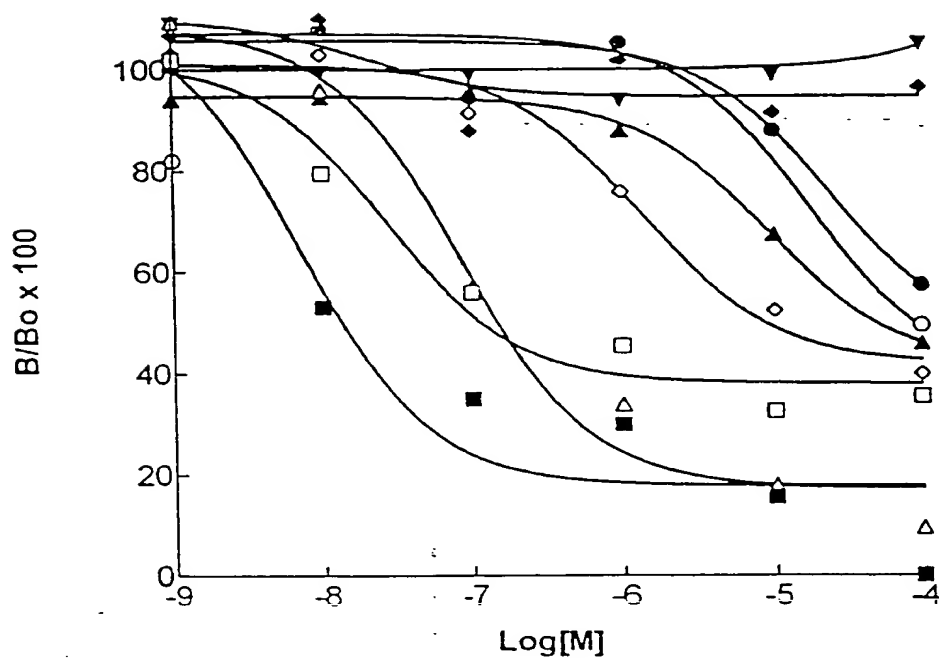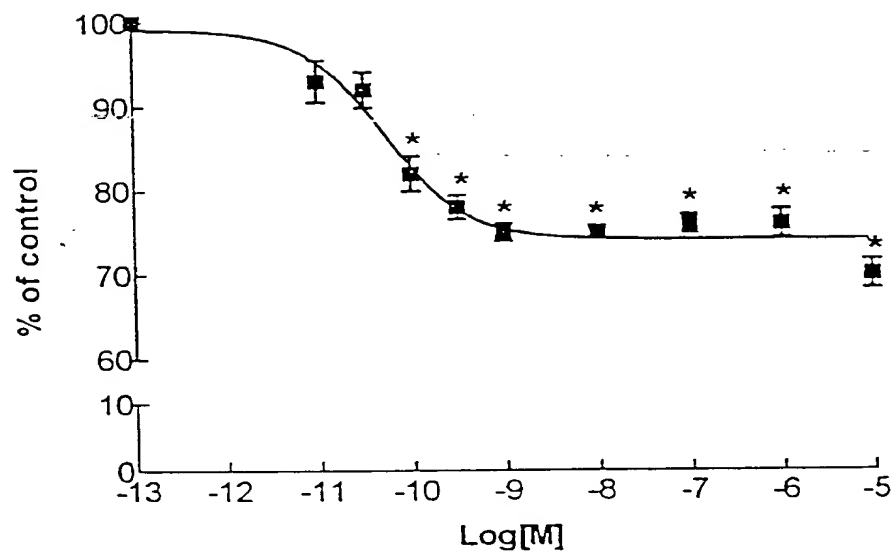FIGURE 3

FIGURE 4
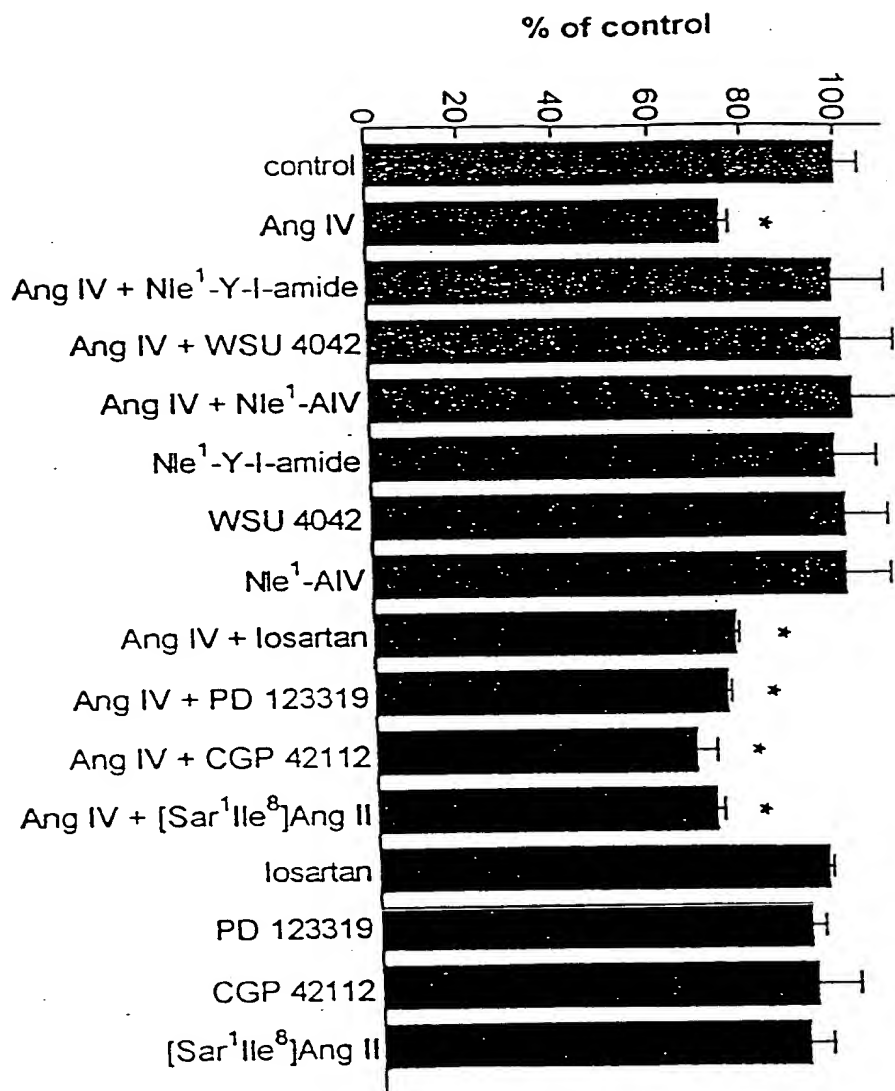
FIGURE 5
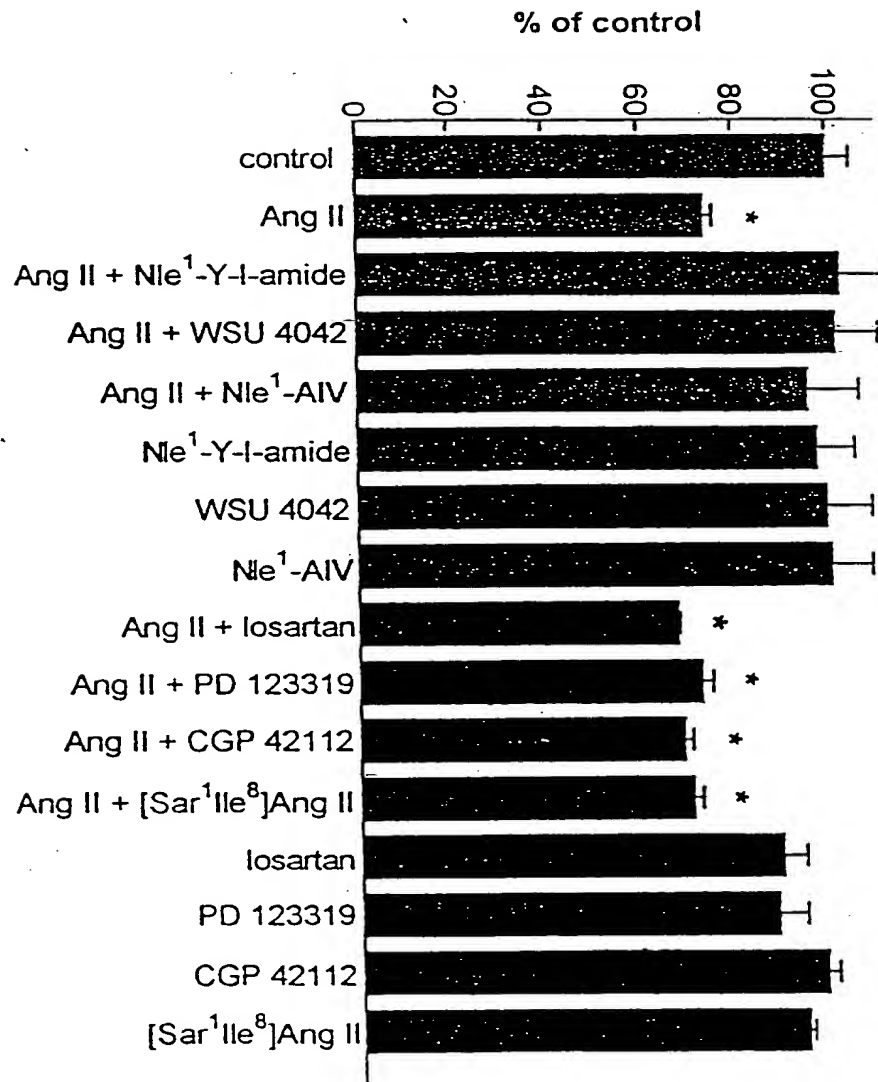
FIGURE 6
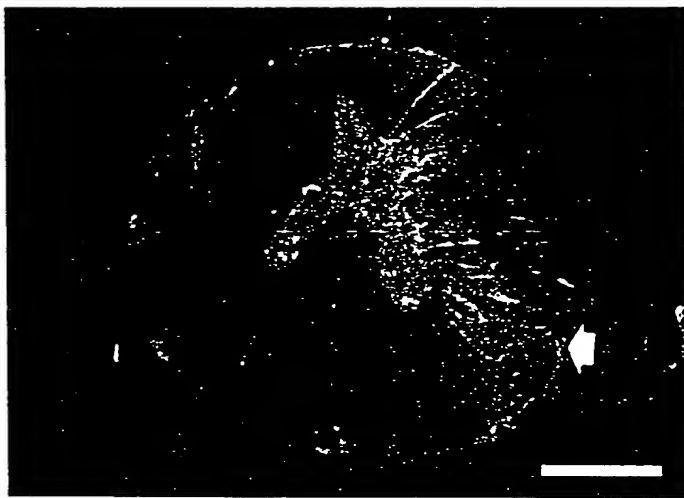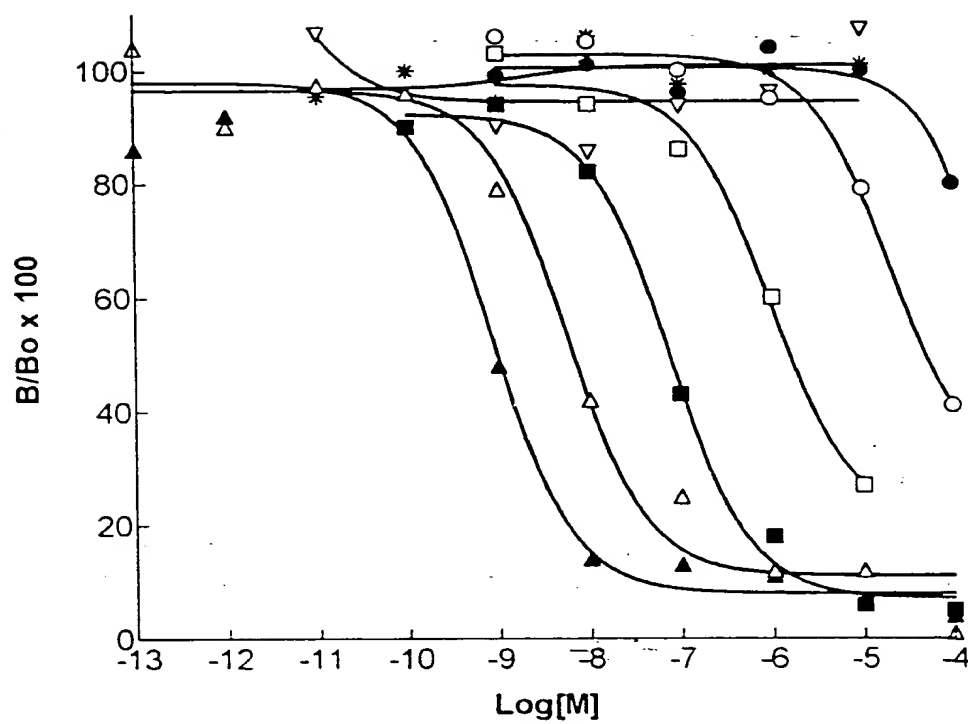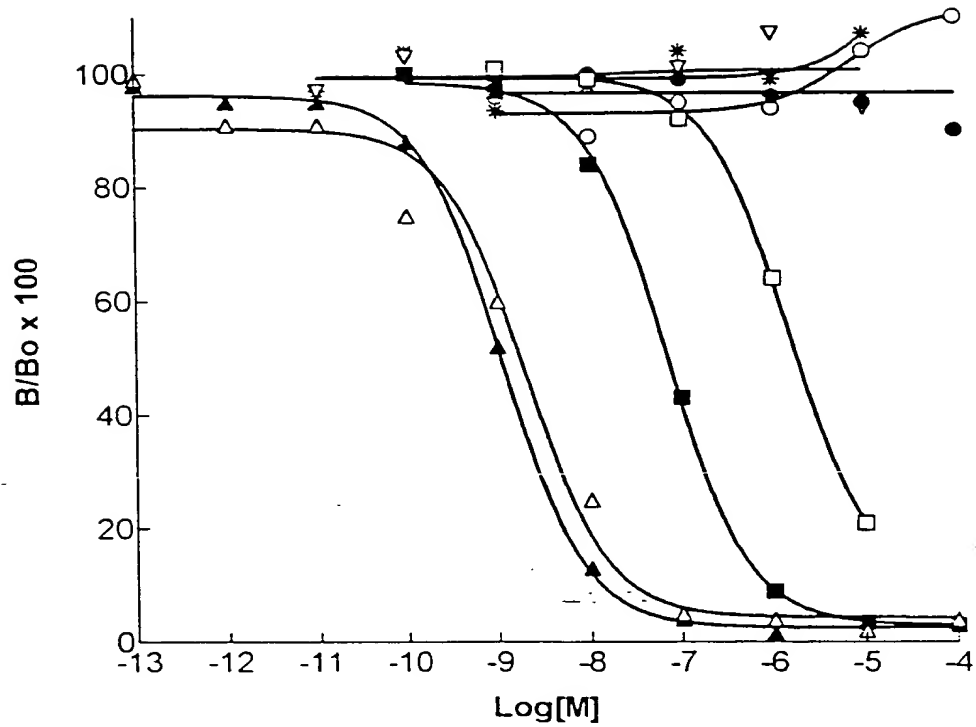
FIGURE 7

FIGURE 8

FIGURE 9

β - Globin Gene Structure

A

Exon 1

H171 ↓ H170 ↑

Exon 2

H172 ↓

Exon 3

H173 ↓

LVVYPWTQRF
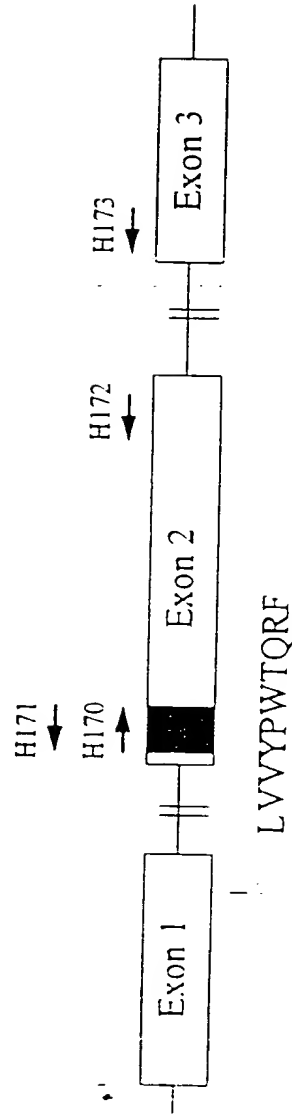
B

Oligonucleotide sequences:

H170: 5' CTGGTTGTCTACCCCTGGACTCAGAG 3'
H171: 5' CTCTGAGTCCAGGGGTAGACAACCAG 3'
H172: 5' CTCAGGATCCACATGCAGCTTATCACAG 3'
H173: 5' CAGCACAACCACTAGCACATTGCC 3'

FIGURE 10

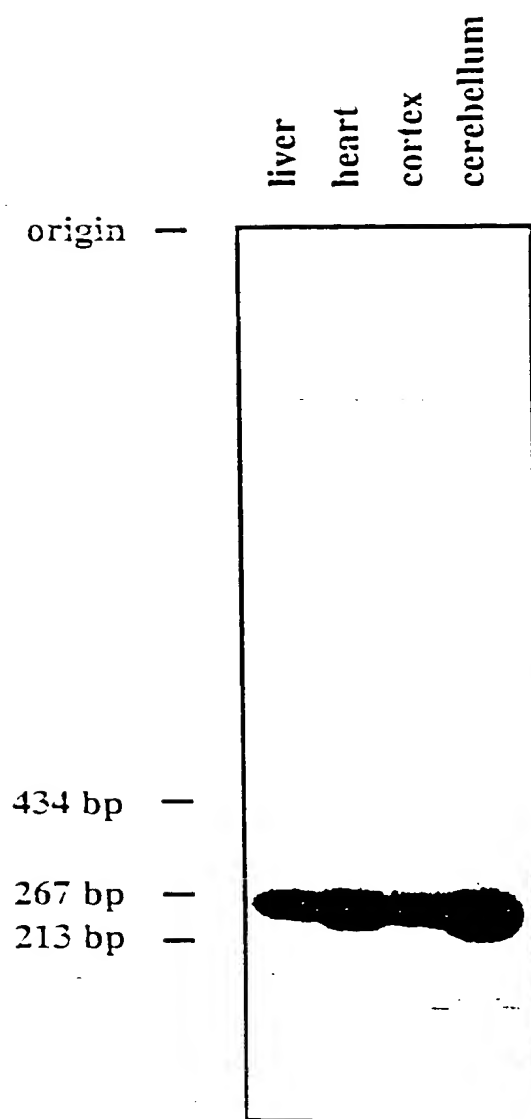FIGURE 11

```
                                          10        20        30
EX                              CACAAACTCAGAAACAGACACCATGGTGCACCTGA
                                ::::::::::::::::::::::::::::::::::::
RNBGLO  TGCTTCTGACATAGTTGTGTTGACTCACAAACTCAGAAACAGACACCATGGTGCACCTGA
              10        20        30        40        50        60


            40        50        60        70        80        90
EX      CTGATGCTGAGAAGGCTGCTGTTAATGGCCTGTGGGGAAAGGTGAACCCTGATGATGTTG
        :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
RNBGLO  CTGATGCTGAGAAGGCTGCTGTTAATGGCCTGTGGGGAAAGGTGAACCCTGATGATGTTG
              70        80        90        100       110       120


            100       110       120       130       140       150
EX      GTGGCGAGGCCCTGGGCAGGCTGCTGGTTGTCTACCCTTGGACCCAGAGGTACTTTGATA
        :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
RNBGLO  GTGGCGAGGCCCTGGGCAGGCTGCTGGTTGTCTACCCTTGGACCCAGAGGTACTTTGATA
              130       140       150       160       170       180


            160       170       180       190       200       210
EX      GCTTTGGGGACCTGTCCTCTGCCTCTGCTATCATGGGTAACCCTAAGGTGAAGGCCCATG
        :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
RNBGLO  GCTTTGGGGACCTGTCCTCTGCCTCTGCTATCATGGGTAACCCTAAGGTGAAGGCCCATG
              190       200       210       220       230       240


            220       230       240       250       260       270
EX      GCAAGAAGGTGATAAACGCCTTCAATGATGGCCTGAAACACTTGGACAACCTCAAGGGCA
        :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
RNBGLO  GCAAGAAGGTGATAAACGCCTTCAATGATGGCCTGAAACACTTGGACAACCTCAAGGGCA
              250       260       270       280       290       300


            280       290       300       310       320       330
EX      CCTTTGCTCATCTGAGTGAACTCCACTGTGACAAGCTGCATGTGGATCCTGAGAACTTCA
        :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
RNBGLO  CCTTTGCTCATCTGAGTGAACTCCACTGTGACAAGCTGCATGTGGATCCTGAGAACTTCA
              310       320       330       340       350       360


            340       350       360       370       380       390
EX      GGCTCCTGGGCAATATGATTGTGATTGTGTTGGGCCACCACCTGGGCAAGGAATTCACCC
        :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
RNBGLO  GGCTCCTGGGCAATATGATTGTGATTGTGTTGGGCCACCACCTGGGCAAGGAATTCACCC
              370       380       390       400       410       420


            400       410       420       430       440       450
EX      CCTGTGCACAGGCTGCCTTCCAGAAGGTGGTGGCTGGAGTGGCCAGTGCCCTGGCTCACA
        :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
RNBGLO  CCTGTGCACAGGCTGCCTTCCAGAAGGTGGTGGCTGGAGTGGCCAGTGCCCTGGCTCACA
              430       440       450       460       470       480


            460       470       480       490       500       510
EX      AGTACCACTAAACCTCTTTTCCTGCTCTTGTCTTTGTGCAATGGTCAATTGTTCCCAAGA
        :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
RNBGLO  AGTACCACTAAACCTCTTTTCCTGCTCTTGTCTTTGTGCAATGGTCAATTGTTCCCAAGA
              490       500       510       520       530       540


            520       530       540       550       560       570
EX      GAGCATCTGTCAGTTGTTGTCAAAATGACAAAGACCTTTGAAAATCTGTCCTACTAATAA
        :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
RNBGLO  GAGCATCTGTCAGTTGTTGTCAAAATGACAAAGACCTTTGAAAATCTGTCCTACTAATAA
              580       590       600       610


            580       590       600       610
EX      AAGGCATTTACTTTCACTGCAAAAAAAAAAAAAAAAAAAA
        :::::::::::::::::::::::
RNBGLO  AAGGCATTTACTTTCACTGC
```

FIGURE 12

```
                                            10        20        30
EX                              CACAAACTCAGAAACAGACACCATGGTGCACCTGA
                                                    M   V   H   L

         40        50        60        70        80        90
EX    CTGATGCTGAGAAGGCTGCTGTTAATGGCCTGTGGGGAAAGGTGAACCCTGATGATGTTG
       T   D   A   E   K   A   A   V   N   G   L   W   G   K   V   N   P   D   D   V

         100       110       120       130       140       150
EX    GTGGCGAGGCCCTGGGCAGGCTGCTGGTTGTCTACCCTTGGACCCAGAGGTACTTTGATA
       G   G   E   A   L   G   R   L   L   V   V   Y   P   W   T   Q   R   Y   F   D

         160       170       180       190       200       210
EX    GCTTTGGGGACCTGTCCTCTGCCTCTGCTATCATGGGTAACCCTAAGGTGAAGGCCCATG
       S   F   G   D   L   S   S   A   S   A   I   M   G   N   P   K   V   K   A   H

         220       230       240       250       260       270
EX    GCAAGAAGGTGATAAACGCCTTCAATGATGGCCTGAAACACTTGGACAACCTCAAGGGCA
       G   K   K   V   I   N   A   F   N   D   G   L   K   H   L   D   N   L   K   G

         280       290       300       310       320       330
EX    CCTTTGCTCATCTGAGTGAACTCCACTGTGACAAGCTGCATGTGGATCCTGAGAACTTCA
       T   F   A   H   L   S   E   L   H   C   D   K   L   H   V   D   P   E   N   F

         340       350       360       370       380       390
EX    GGCTCCTGGGCAATATGATTGTGATTGTGTTGGGCCACCACCTGGGCAAGGAATTCACCC
       R   L   L   G   N   M   I   V   I   V   L   G   H   H   L   G   K   E   F   T

         400       410       420       430       440       450
EX    CCTGTGCACAGGCTGCCTTCCAGAAGGTGGTGGCTGGAGTGGCCAGTGCCCTGGCTCACA
       P   C   A   Q   A   A   F   Q   K   V   V   A   G   V   A   S   A   L   A   H

         460       470       480        490       500       510
EX    AGTACCACTAAACCTCTTTTCCTGCTCTTGTCTTTGTGCAATGGTCAATTGTTCCCAAGA
       K   Y   H   *

         520       530       540       550       560       570
EX    GAGCATCTGTCAGTTGTTGTCAAAATGACAAAGACCTTTGAAAATCTGTCCTACTAATAA

         580       590        600       610
EX    AAGGCATTTACTTTCACTGCAAAAAAAAAAAAAAAAAAA
```

FIGURE 13